Diffusion, Patching, and Capping of Stearoylated Dextrans on 3T3 Cell Plasma Membranes†

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ABSTRACT: Fluorescence-labeled trinitrophenylated stearoylated dextrans have been used as controllable analogues of cell membrane proteins on model membranes and on a variety of natural cell membranes. This paper reports their behavior on 3T3 mouse fibroblast plasma membranes. Spatial distribution on the membrane was studied by fluorescence microscopy, and molecular mobility was measured by fluorescence photobleaching recovery. At concentrations from 10^2 to 3×10^3 molecules/μm^2 essentially homogeneous fluorescence was observed after treatment with these stearoyldextrans in culture. Diffusion coefficients and fractional recovery of fluorescence after photobleaching were concentration independent. For 3×10^3 molecules/μm^2 we found at 23 °C $D = (3.0 \pm 1.8) \times 10^{-10}$ cm^2/s with 65 ± 17% recovery and at 37 °C $D = (7.0 \pm 5.0) \times 10^{-10}$ cm^2/s without a change of the fractional recovery. Cross-linking with antibodies stopped diffusion on a macroscopic scale and sometimes induced patching, mottling (defined as the development of gaps in the fluorescence layer), and capping (defined as the confinement of the fluorescence to less than 50% of the cell). Capping required ~3 h at 37 °C and was inhibited by metabolic poisons and cytochalasin B. These drugs did not affect stearoyldextran diffusion or fractional recovery. Colchicine, which did not dramatically affect capping, slowed diffusion two- to threefold but did not affect fractional recovery. The antibody inhibition of the diffusion of stearoyldextrans precedent to capping did not affect the diffusion of a lipid probe or fluorescein isothiocyanate labeled membrane proteins. When the trinitrophenylated stearoyldextran was cleared from most of the surface by capping and the surface subsequently relabeled with stearoyldextran, the diffusion coefficient and fractional recovery of the second label were identical with those of the first label prior to capping. Thus, capping does not clear an immobilizing factor from the membrane.

The study of the lateral mobility of cell membrane components has been pursued by two differing approaches: (1) the study of the redistribution of membrane components by cross-linking with external ligands (Taylor et al., 1971; Nicolson, 1976a,b; Schreiner & Unanue, 1976) and (2) the study of the restoration of concentration equilibrium to regions of induced concentration gradients (Frye & Edidin, 1970; Poo & Cone, 1974; Edidin et al., 1976; Jacobson et al., 1976; Schlessinger et al., 1976b; Poo & Jaffe, 1977).

Taylor et al. (1971) showed by fluorescence that surface immunoglobulin initially homogeneously distributed on the B lymphocyte surface became redistributed into small clusters called "patches" after cross-linking with antibodies. This process was found to be independent of cell metabolism and cytoskeletal integrity. At 37 °C in the presence of metabolic energy and microfilaments, the patches were found to undergo a driven collective motion which caused them to become confined to a "cap" at one pole of the cell. Patching and capping have since been observed for a variety of receptors on a variety of cells (Nicolson, 1976a,b; Schreiner & Unanue, 1976).

Several experimenters have taken the approach of inducing concentration gradients by various means. Among these are heterokaryon fusion (Frye & Edidin, 1970), bleaching of absorbance (Poo & Cone, 1974), and electrophoresis (Poo & Jaffe, 1977). A widely used and highly quantitative technique, fluorescence photobleaching recovery (FPR),[1] has recently been developed (Axelrod et al., 1976; Edidin et al., 1976; Jacobson et al., 1976; Schlessinger et al., 1976b). In this technique the membrane component to be studied is fluorescently labeled, and its fluorescence from a small spot on the membrane (excited by a focused laser beam) is monitored. A brief increase of laser intensity causes some of the fluorescence to be bleached, and subsequent recovery due to motions of the molecules is measured. FPR experiments have shown that some, but not all, membrane components are capable of passive diffusion, the mean motion of macromolecules that results from their random Brownian motion. In particular, lipid molecules have been found to be totally free to diffuse (with $D \simeq 10^{-8}$ cm^2/s), while protein molecules have been found to diffuse more slowly, typically with $D \simeq 10^{-10}$ cm^2/s except for a nondiffusing fraction. The constraints on protein diffusion and the cause of the nondiffusing fraction remain essential questions.

The mechanisms of the driven collective motions of cell membrane components are even less clearly understood. A number of hypotheses have been proposed to explain the most studied of these motions, capping. Two basic classes of models have emerged: (1) models which propose that capping results from hydrodynamic drag on membrane proteins induced by lipid flow (Bretscher, 1976) and (2) models which propose that capping results from a contraction of microfilaments, which

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[1] Abbreviations used: FPR, fluorescence photobleaching recovery; BLM, black lipid membranes; diI, 3,3′-dioctadecylindocarbocyanine iodide; FITC, fluorescein isothiocyanate; DMEM, Dulbecco's modified Eagle's medium; SD, stearoyldextran; TNP, trinitrophenyl; TSD, TNP-stearoyldextran; AcRSD, acetylrhodamine–stearoyldextran; AcFSD, acetylfluorescein–stearoyldextran; AcT_5RSD, Ac-5-TNP-rhodamine–stearoyldextran; AcT_5FSD, Ac-5-TNP-fluorescein–stearoyldextran; TRSD, TNP-rhodamine–stearoyldextran; TFSD, TNP-fluorescein–stearoyldextran; AcRD, acetylrhodamine–dextran; TRD, TNP-rhodamine–dextran; TSD, TNP-stearoyldextran; BSS, buffered saline solution.

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Table I: Composition of Stearoyldextrans

name	designation	approx no. of groups per dextran molecule				
		fatty acid[a]	TNP	rhodamine	fluorescein	acetyl
stearoyldextran	SD	1.4	0	0	0	0
TNP-stearoyldextran	TSD	1.3	40	0	0	0
acetylrhodamine-stearoyldextran	AcRSD	1.3	0	1	0	0
acetylfluorescein-stearoyldextran	AcFSD	1.3	0	0	1	0
Ac-TNP(5)-rhodamine-stearoyldextran	AcT_5RSD	1.3	5–20	1	0	20–35
Ac-TNP(5)-fluorescein-stearoyldextran	AcT_5FSD	1.3	5–20	0	1	20–35
TNP-rhodamine-stearoyldextran	TRSD	1.3	40	1	0	0
TNP-fluorescein-stearoyldextran	TFSD	1.3	40	0	1	0
acetylrhodamine-dextran	AcRD	1.3	0	1	0	40
TNP-rhodamine-dextran	TRD	1.3	40	1	0	0

[a] Roughly 50% stearic and 50% palmitic acid. These are mean values for dextran derivative preparation. It is likely that the membrane will select for molecules with higher numbers of fatty acids.

are hypothesized to be linked initially to membrane proteins or to become so on ligand binding, cross-linking, or patching (dePetris & Raff, 1973; Schreiner & Unanue, 1976).

An understanding of the motions of membrane components and the phenomena of patching and capping would be facilitated by the study of simple model membrane systems. Such an approach was pursued by Wolf et al. (1977), who incorporated fluorescently labeled trinitrophenylated stearoylated dextrans, as analogues of membrane receptors, into planar black lipid membranes (BLM) and studied the effects of cross-linking ligands on receptor diffusion and distribution. Their system facilitated the study of membrane macromolecules in the absence of the cytoskeleton and cell metabolism. They found that cross-linking can result in receptor immobilization and "patching".

Recently, P. Henkart and C. Neels (unpublished experiments) incorporated these synthetic antigens into the plasma membranes of a variety of cells and found that on cross-linking with antibodies these antigens will both patch and cap on many cells. The capping they observed, like that of natural membrane receptors, was found to be a metabolically active process which required the integrity of the microfilaments.

Stearoyldextrans could provide us with a controllable system where both diffusion and capping could be studied simultaneously. In this paper we report on the results of such a study. We have studied the diffusion by FPR, and antibody-induced redistribution, by fluorescence microscopy, of stearoylated dextrans on 3T3 cell plasma membranes.

Materials and Methods

(*1*) *Reagents.* (*a*) *Antibodies.* Affinity-pure IgG antibodies to TNP were obtained from the serum of a sheep hyperimmunized to TNP-hemocyanin. The antibodies were purified by sulfate precipitation, affinity purification of DNP-Lys–Sepharose, and gel filtration on Sephadex G200. The association constant of these antibodies with DNP-Lys determined by measuring the quenching of intrinsic antibody fluorescence upon hapten binding (Velick et al., 1960) was 1.6×10^7 M^{-1}. The IgG fractions of rabbit antisheep IgG were obtained from Miles Yeda. Rhodamine- and fluorescein-conjugated rabbit antisheep IgG was obtained from Cappel. These preparations were used without further purification. $(Fab')_2$ fragments of purified anti-TNP antibodies were obtained by digestion with pepsin (Edelman & Marchalonis, 1967), followed by gel filtration on Sephadex G150. The Fab′ fragments were prepared from the $(Fab')_2$ preparation by reduction and alkylation (Edelman & Marchalonis, 1967). These preparations were labeled with rhodamine isothiocyanate obtained from Curtin Matheson or fluorescein isothiocyanate (FITC) obtained from Sigma Chemical Co. This FITC was also used for nonspecific cell labeling.

(*b*) *Lipid Probe.* 3,3′-Dioctadecylindocarbocyanine iodide (diI) was used as a probe of lipid diffusion. It was the generous gift of A. S. Waggoner. This probe has been used as a lipid probe in various cell membranes (Sims et al., 1974; Schlessinger et al., 1976a; Axelrod et al., 1978) and recently in BLM (Fahey et al., 1976; Fahey & Webb, 1978).

(*c*) *Stearoyldextrans.* The preparation of stearoyldextrans has been described extensively elsewhere (P. Henkart and C. Neels, unpublished experiments; Wolf et al., 1977). The composition of the stearoyldextrans used in this paper and the coding used to identify them are tabulated in Table I.

(*d*) *Media and Buffers.* The buffers and tissue culture media, Hanks' BSS, Dulbecco's modified Eagles' medium (DMEM), DMEM with Hepes, DMEM with Hepes without phenol red, and fetal calf serum were obtained from Grand Island Biological Co. When used in the presence of antibodies, the fetal calf serum was inactivated.

(*2*) *Cells.* Mouse 3T3 cells were grown in 35-mm petri dishes (Corning 25000) in 2 mL of DMEM with 10% fetal calf serum at 37 °C in a water-saturated, 10% CO_2, 90% air atmosphere. Experiments were made with cells at 20–50% confluency, 1 to 2 days after plating. The petri dishes result in background fluorescence from two sources, fluorescence of the plastic and fluorescence of exogeneous label bound nonspecifically to the plastic. In these experiments this combined background was low when compared to the fluorescence, of labeled cells, 5–10% at 476 nm and ≤1% at 514, 531, and 568 nm.

(*3*) *Cell Labeling.* (*a*) *Stearoyldextran and Antibody Labeling.* Cells were labeled with stearoyldextrans and then with antibodies by the following procedure, except as noted in the text. (1) Cells were washed gently 3 times with 2 mL of DMEM in ~1 min. (2) One milliliter of DMEM was added to the dish, and then 5–50 μL of stearoyldextran solution (0.2–2 mg/mL in Hanks' BSS) was added. (3) This was incubated for 15 min at 37 °C in the CO_2 incubator. (4) Cells

were washed gently 3 times with 2 mL of DMEM. (5) One milliliter of DMEM with Hepes buffer and 5–50 μL of antibody solution (2.2 mg/mL in phosphate-buffered saline) were added to the dish. (6) This was incubated for 30 min at room temperature. (7) Cells were washed gently 3 times with 2 mL of DMEM. In antibody labeling experiments we define this as time $t = 0$. (8) One milliliter of medium was added to the dish, DMEM if further incubations were to be made at 37 °C and DMEM with Hepes if they were to be made at room temperature. Medium without phenol red, which is itself a fluorescent dye, was used if the cells were to be observed during subsequent incubation. (9) Cells were incubated at 37 °C in the CO_2 incubator or in the air at room temperature for the desired amount of time. (10) Cells were washed gently 3 times with 2 mL of Hanks' BSS and then observed in 1.5 mL of Hanks' BSS. When observations were made at an earlier time after (3) or (6), these steps were followed immediately by (10). In general, cells were discarded after observation (and not returned to the incubator). Treatment with Hanks' BSS, however, did not affect the ability of cells to patch and cap. Surface concentrations of stearoyldextrans were determined from surface fluorescence intensities as described by Wolf et al. (1977).

(*b*) *diI Labeling.* To label cells with diI, we added 20 μL of a 0.38 mg/mL solution of diI in EtOH to 2 mL of Hanks' BSS. This was incubated for 8 min at 37 °C and then washed 3 times in Hanks' BSS. Because of internalization, diI labeling was always done last, and measurements made within 0.5 h.

(*c*) *FITC Labeling.* Cell membranes were nonspecifically labeled with FITC by the method of Schlessinger et al. (1976a). Cells were incubated for 10 min at 37 °C with 1 mL of Hanks' BSS containing 20 μg of FITC and then washed 5 times with Hanks' BSS. FITC labeling preceded stearoyldextran labeling.

(*4*) *Instrumentation.* Our apparatus and the technique of FPR have been extensively described elsewhere (Axelrod et al., 1976; Koppel et al., 1976; Wolf et al., 1977). In FPR the exogenous fluorescence from a small spot epiilluminated by a focused laser beam on a cell is monitored. For these experiments a 1.1 μm radius beam was used. The spot was briefly exposed to bleaching intensities of incident light, and the recovery of fluorescence after bleaching was observed by using a 40X water immersion phase objective. Bleaching times were 6–150 ms and laser powers 0.1–10 mW. For conditions used in these experiments, localized heating at the membrane due to the bleaching pulse is in the worst possible case <0.2 °C (Axelrod, 1977). Furthermore, these bleaching conditions have been shown not to introduce photoinduced artifacts in the measurements (D. E. Wolf, M. Edidin, and P. R. Dragsten, unpublished experiments).

A heated stage for 37 °C was prepared as follows: A coil of nichrome wire was sandwiched between two microscope slides. The upper slide was half the length of the lower and was centered on the lower. The sandwich was sealed with epoxy. The petri dish was thermally sealed to the upper slide with Dow heat conducting silicone grease. The temperature was monitored with a Bailey Instruments Co. BAT-8 digital thermometer with a wire thermistor taped to the microscope objective to measure the temperature in the medium just above the point of observation. The temperature was controlled by hand with a Lambda LL-901-OV regulated power supply. In this manner we were able to control the temperature to within 2 °C.

(*5*) *Data Analysis.* FPR data were analyzed by the half-time method discussed in Axelrod et al. (1976). For these

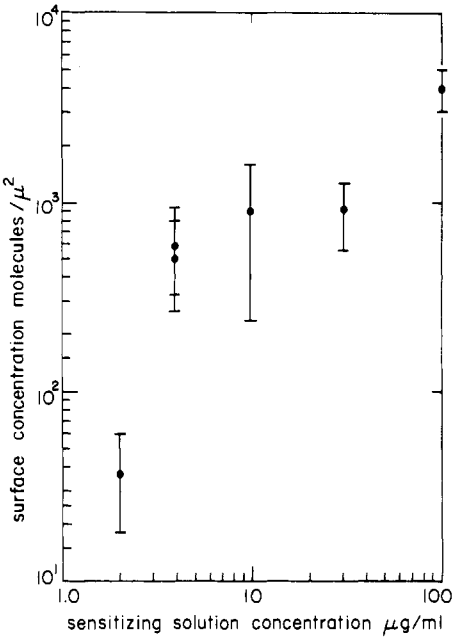

FIGURE 1: Adsorption of AcT_5RSD to 3T3 cell plasma membranes.

experiments the diffusion of AcT_5RSD on 3T3 cells at 3×10^3 molecules/μm² was standardized against the diffusion of TRSD at 10^4 molecules/μm² on EPC BLM dispersed in *n*-octane (Wolf et al., 1977). (See Table I for coding of stearoyldextran preparations.) The diffusion coefficient of AcT_5RSD of 3T3 cells was then used as a reference standard for other cell experiments. Values of $t_{1/2}$ and the mobile fraction measured were obtained by averaging measurements from at least 10 cells generally with two bleaches at each spot on a cell and compared to this standard. This method of standardization corrected for variations in beam radius of 0.2–0.3 μm, which occurred over long periods as a result of modification of laser source and apparatus.

We have not observed any differences in stearoyldextran diffusion associated with cell shape. However, since there is a tendency when doing FPR measurements to choose flat cells and since rounded, less adherent cells may be removed in washing, our data may be biased in favor of cells in interphase.

Results

(*1*) *Adsorption of Stearoyldextrans to 3T3 Cell Plasma Membranes.* Immediately following labeling with stearoyldextrans from the aqueous medium, the resulting fluorescence was essentially homogeneous and diffuse over the cell, although some structures, such as the perinuclear ruffled membrane on flat cells, did label somewhat more intensely (see Figure 4A,B). The resulting surface concentration as a function of initial sensitizing solution concentration is shown in Figure 1. This adsorption isotherm shows a saturating concentration of (5–6) $\times 10^3$ molecules/μm² and an association constant K_a of $\sim 10^6$ M^{-1}. This saturation limit is similar to that observed on BLM, while K_a is an order of magnitude less (Wolf et al., 1977). In the majority of the experiments to be described here, cells at 20–50% confluency were sensitized to 1 mL of DMEM with 100 μg of stearoyldextran for 15 min at 37 °C. These conditions resulted in a surface concentration of $(3 \pm 1) \times 10^3$ molecules/μm². This concentration is about 50 or 60% of saturation and is equal to about 10% of the protein concentration.

As was the case with BLM, stearoyl groups were essential for the binding of stearoyldextrans to cells. AcRD showed only nonspecific staining of ≤2% of the labeling of 3T3 cells

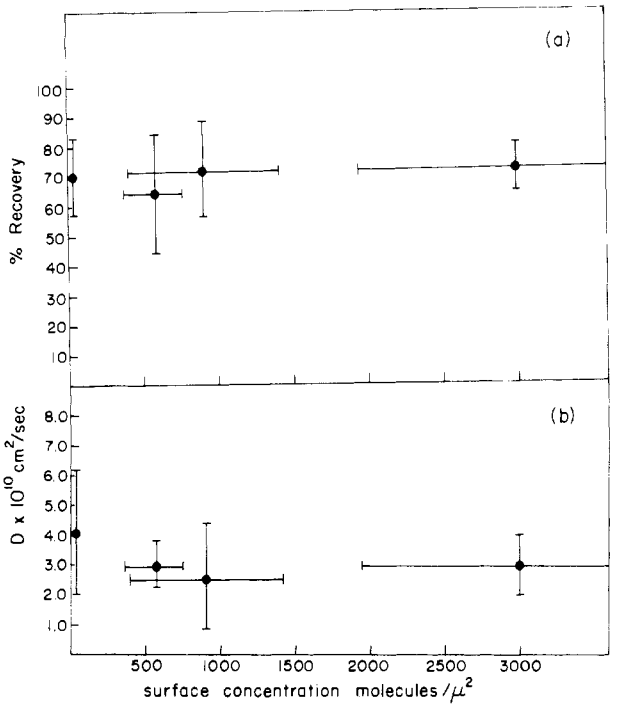

FIGURE 2: Diffusion and fractional recovery of AcT_5RSD on 3T3 cell plasma membranes as a function of surface concentration. (a) shows the fractional recovery; (b) shows the diffusion coefficient.

with AcRSD, showing that stearoyl groups are essential for incorporation of stearoyldextrans into the membranes. TNP groups, on the other hand, had no effect on the binding of stearoyldextrans to 3T3 cells; AcRSD, AcT_5RSD, and TRSD all bound equally.

Several pieces of evidence point directly to the conclusion that the observed fluorescent label was largely on the surface during these experiments and not endocytosed into the cytoplasm: (1) A dominant feature of cells with internalized label is negative staining of the nucleus. Such negative staining was not observed with stearoyldextrans until several hours after labeling. (2) On rounded cells the fluorescence can only be brought into focus at the two plasma membranes. (3) After binding to cells, the majority of the stearoyldextran was still accessible to binding by antibodies which are not rapidly internalized. As we shall discuss further below, some endocytosis did occur after ~1 h, but even at 3 h most of the material remained on the surface.

(*2*) *Diffusion of Stearoyldextrans on 3T3 Cell Plasma Membranes.* The two-dimensional diffusion coefficients for AcT_5RSD bound to 3T3 cell plasma membranes at room temperature are shown as a function of surface concentration in Figure 2. These measured surface concentrations were obtained by varying the sensitizing solution conentration, as discussed above. Notice that within the limits of experimental uncertainty the diffusion coefficient was independent of surface concentration. In Figure 2a we show the fractional recovery as a function of surface concentration. The recovering fraction showed no dependence on surface concentration. At a concentration of 3×10^3 molecules/μm^2, we found that AcT_5RSD showed $D = (3.0 \pm 1.8) \times 10^{-10}$ cm^2/s with $65 \pm 17\%$ recovery. These numbers were derived from 113 measurements from experiments done on 11 separate days. Within each experimental set, a 33% variation in D was typical. This concentration is typical of cell membrane proteins and invites comparison with available data for protein diffusion on 3T3 cells (see Table II). We find that stearoyldextrans show diffusion rates and fractional recoveries which are very similar to those observed for membrane proteins.

Table II: Diffusion on 3T3 Cells

probe	D (cm^2/s)	% recovery	reference
diI	$(5\text{–}10) \times 10^{-9}$	83 ± 10	Reidler (personal communication), Eldridge (personal communication)
Fl-GM1 (fluorescent ganglioside)	$(2\text{–}5) \times 10^{-9}$[a]	90 ± 10	Reidler (personal communication), Eldridge (personal communication)
rhodamine S Con A	$(3.9 \pm 1.1) \times 10^{-11}$		Schlessinger et al. (1977a)
rhodamine S Con A	$(5.2 \pm 1.7) \times 10^{-10}$	64 ± 14	Eldridge (personal communication)
rhodamine	$(2.6 \pm 0.8) \times 10^{-10}$	50 ± 10	Schlessinger et al. (1977a), Eldridge (personal communication)
fluorescein CSP or rhodamine anti-CSP	$< 5 \times 10^{-12}$		Schlessinger et al. (1977b)

[a] Both Reidler and Eldridge found that D(diI) = 2 × D(GM1).

As illustrated in Table III, AcRSD, AcT_5RSD, and TRSD all exhibit the same diffusion coefficient and mobile fraction. Thus these parameters are independent of the number of TNP groups. Furthermore, comparison of data for AcT_5RSD and AcT_5FSD shows that changing fluorophores did not alter the behavior of the molecule. These results are consistent with results obtained on BLM (Wolf et al., 1977).

Increasing the temperature to 37 °C increased D to $(7 \pm 5) \times 10^{-10}$ cm^2/s but had no effect on the fractional recovery. These results are consistent with results reported by Schlessinger et al. (1976) and Axelrod et al. (1978) on the effect of temperature on the diffusion of cell membrane proteins and lipids.

We have tested whether the incomplete recoveries observed were due to the internalization of a fraction of the stearoyldextran, by first labeling with AcT_5FSD at 3×10^3 molecules/μm^2 and then with rhodamine-labeled Fab′ fragments of anti-TNP IgG (50 μg in 1 mL for 10 min at 37 °C). The rhodamine Fab′ fragments should recover more completely than stearoyldextran alone if part of the AcT_5FSD were internalized. On the contrary, they both recovered equally and with the same diffusion coefficients.

To further test whether endocytosis is a possible cause of incomplete recovery, we pretreated cells for 1–2 h with metabolic poisons and inhibitors of endocytosis. Neither 10^{-2} M NaN_3, 10^{-2} M NaN_3 and 10^{-2} M 2-deoxy-D-glucose, nor 10^{-2} M NaN_3, 10^{-2} M 2-deoxy-D-glucose, and 10^{-2} M NaF had any effect on either the diffusion coefficient or the fractional recovery.

To consider whether microfilaments or microtubules have any influence on stearoyldextran diffusion, we studied the effect of cytochalasin B (10 μg/mL in 1% Me_2SO) and colchicine (10^{-4} M) on the diffusion of AcT_5RSD at 3×10^3 molecules/μm^2. Cells were pretreated with the drug for 1 h before labeling with AcT_5RSD and then continuously exposed to it. No effect was observed for cytochalasin B on either the diffusion coefficient or the fractional recovery. Colchicine decreased D two- to threefold but had no effect on the mobile fraction.

(*3*) *Clearing of Stearoyldextrans from 3T3 Cell Plasma Membranes.* When 3T3 cells labeled with 3×10^3 mole-

Table III: Diffusion on 3T3 Cell Plasma Membranes at Room Temperature

fluorescent probe	treatment of cells	diffusion coeff ± SD (cm^2/s)	% recovery
AcT_5RSD	2 μg/mL, 15 min, 37 °C, and washed; yields ~40 molecules/μm^2	$(4.0 \pm 2.1) \times 10^{-10}$	72 ± 13
AcT_5RSD	10 μg/mL, 15 min, 37 °C, and washed; yields ~3.0×10^3 molecules/μm^2	$(3.0 \pm 1.8) \times 10^{-10}$	65 ± 17
AcRSD	100 μg/mL, 15 min, 37 °C, and washed; yields ~3.0×10^3 molecules/μm^2	$(3.0 \pm 1.6) \times 10^{-10}$	62 ± 10
TRSD	100 μg/mL, 15 min, 37 °C, and washed; yields ~3.0×10^3 molecules/μm^2	$(2.6 \pm 2.1) \times 10^{-10}$	76 ± 11
AcT_5FSD	100 μg/mL, 15 min, 37 °C, and washed	$(3.0 \pm 2.0) \times 10^{-10}$	65 ± 17
AcT_5RSD	100 μg/mL, 15 min, 37 °C, and washed; 5.5 μg/mL anti-TNP	$(9.1 \pm 4.1) \times 10^{-11}$	35 ± 21
AcT_5RSD	100 μg/mL, 15 min, 37 °C, and washed; 22 μg/mL anti-TNP	$\sim 11 \times 10^{-11}$ [a]	4 ± 7
AcT_5RSD	100 μg/mL, 15 min, 37 °C, and washed; 110 μg/mL anti-TNP	$\sim 11 \times 10^{-11}$ [a]	12 ± 11
AcT_5RSD	100 μg/mL, 15 min, 37 °C, and washed; 55 μg/mL anti-TNP, 30 min, room temperature, and washed		11 ± 9
TRSD	100 μg/mL, 15 min, 37 °C, and washed; 55 μg/mL anti-TNP, 30 min, room temperature, and washed		15 ± 8
diI	20 μL of diI solution (a 38 mg/mL in EtOH) to 2 mL of Hanks' BSS, 8 min, 37 °C, and washed	$(6 \pm 3) \times 10^{-9}$	82 ± 14
diI	100 μg/mL TSD, 15 min, 37 °C, and washed; 20 μL of diI solution to 2 mL of Hanks' BSS, 8 min, 37 °C, and washed	$(5 \pm 2) \times 10^{-9}$	88 ± 12
diI	100 μg/mL TSD, 15 min, 37 °C, and washed; 55 μg/mL anti-TNP, 30 min, room temperature, and washed; 20 μL of diI solution to 2 mL of Hanks' BSS	$(7 \pm 3) \times 10^{-9}$	86 ± 10
AcRSD	100 μg/mL AcT_5FSD, 15 min, 37 °C, and washed; 55 μg/mL anti-TNP, 30 min, room temperature, washed, capped by incubating for 3 h at 37 °C, and incubated with 100 μg/mL AcRSD, 15 min, 37 °C	$(3.0 \rightarrow 1.0) \times 10^{-10}$ (off caps) (on caps)	65 ± 9 ~0
FITC	20 μg/mL, 10 min, 37 °C, and washed	$(7 \pm 3) \times 10^{-10}$	62 ± 10
FITC	20 μg/mL, 10 min, 37 °C, and washed; 100 μg/mL AcT_5RSD, 15 min, 37 °C, and washed	$(8 \pm 3) \times 10^{-10}$	52 ± 11
FITC	20 μg/mL, 10 min, 37 °C, and washed; 100 μg/mL AcT_5RSD, 15 min, 37 °C, and washed; 55 μg/mL anti-TNP, 30 min, room temperature, and washed	$(6 \pm 3) \times 10^{-10}$	51 ± 12

[a] These values are approximate, since it is very difficult to measure half-times for small % recoveries and since they represent only a few measurements.

cules/μm^2 of stearoyldextran were continuously incubated at 37 °C in DMEM without fetal calf serum, small submicroscopic fluorescent spots developed after ~1 h. At 3 h on flat cells these spots tended to be collected into a perinuclear necklace. Such structure and localization are characteristic of endocytotic vacuoles. This is illustrated in Figure 3. To show that the bright spots are indeed internalized, we labeled cells with 3×10^3 molecules/μm^2 of AcT_5FSD and incubated them for 3 h at 37 °C. The cells were then fixed for 30 min with 2.5% paraformaldehyde at room temperature and then labeled for 15 min at 37 °C with 50 μg of rhodamine-labeled anti-TNP IgG in 1 mL of medium. The fluorescein fluorescence from AcT_5FSD showed the bright spot necklace against the homogeneous background of fluorescein fluorescence. The rhodamine fluorescence from IgG, which could only bind the fraction of AcT_5FSD that was accessible on the cell surface, showed no necklace; instead, it showed essentially homogeneous surface staining with very few bright spots. After ~15 h most of this fluorescence had also been internalized, although the remains of the original homogeneous surface label were still visible.

This endocytotic process occurred more slowly at room temperature. It occurred more rapidly when fetal calf serum was added to the medium. In order to test whether this latter effect was due to a dextran aggregating component of the fetal calf serum, we preadsorbed the fetal calf serum with Sephadex, itself a dextran. This pretreatment had no effect on the enhancement.

Treatment of the cells with 10 μg/mL cytochalasin B in 1% Me_2SO or 10^{-4} M colchicine had no effect on this endocytotic process. It was inhibited by metabolic poisons, but a high dosage of a combination of poisons, 10^{-2} M NaN_3, 10^{-2} M NaF, and 10^{-2} M 2-deoxy-D-glucose, was required for thorough inhibition. This result is consistent with known requirements for the inhibition of endocytosis in fibroblasts (Silverstein et al., 1977; Steinman et al., 1972).

(*4*) *Effects of Cross-Linking Membrane-Bound Stearoyldextrans with Anti-TNP Antibodies.* (*a*) *Effect on Stearoyldextran Distribution.* Cross-linking of stearoyldextrans on 3T3 cells with anti-TNP antibodies resulted in redistribution phenomena similar to the patching and capping of natural receptors on cell membranes after cross-linking with antibodies or concanavalin A. These redistributions are shown in Figure 4 for AcT_5RSD at 3×10^3 molecules/μm^2. Figure 4A,B shows essentially homogeneous and diffuse fluorescence which follows the initial labeling of the cells with AcT_5RSD. Incubation with anti-TNP IgG at 55 μg in 1 mL of medium caused the fluorescence to become somewhat granular or patchy, par-

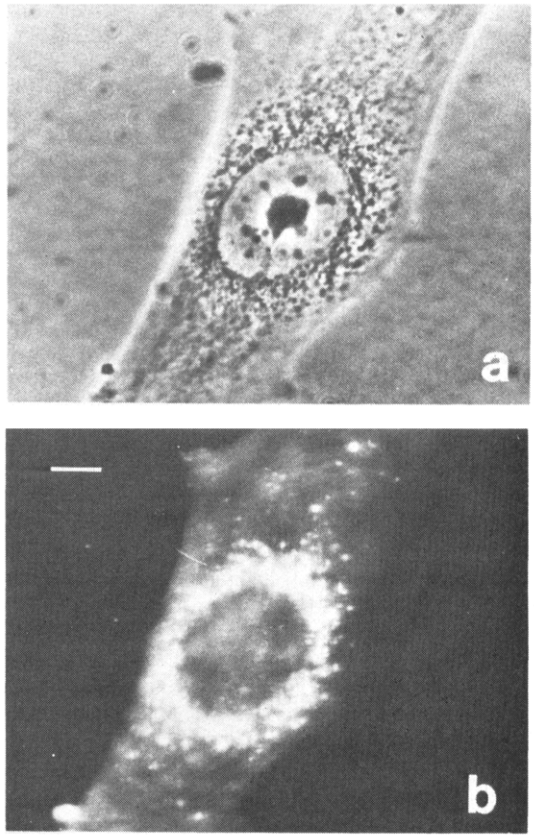

FIGURE 3: Endocytosis of stearoyldextrans by 3T3 cells. Cells were exposed to AcT_5RSD at 100 μg/mL for 15 min at 37 °C and then washed. This resulted in essentially homogeneous staining as shown in Figure 4a,b. Cells were then incubated for 3 h at 37 °C. While homogeneous membrane fluorescence is still present, a necklace of brightly fluorescent endocytotic vacuoles surrounds the nucleus. (a) Phase; (b) fluorescence. The bar = 10 μm.

ticularly on weakly labeled cells. Some of these fluorescent "micropatches" were too small for size resolution, but spacings could be resolved. This graininess developed during the first 5 min of incubation. After ~30 min some of the patches enlarged and the fluorescence on some cells, particularly flat ones, became mottled with dark spots devoid of fluorescence. We define this phenomenon as mottling. Patching and mottling are illustrated in Figure 4C,D. Mottling is most clearly defined and observed on flatter cells. The patching and mottling in Figure 4C,D were very similar in appearance to those observed on BLM (Wolf et al., 1977). At this stage some cells showed early signs of capping as evidenced by the absence of fluorescence from peripheral regions of the cell. After the unadsorbed antibodies were washed away, the cells were returned to 37 °C for further incubation. With time, AcT_5RSD was seen to cap. After 2.5 h at 37 °C, 80% of the cells were seen to have capped as defined by fluorescence being confined to 70% of the cell (see Figure 4E–H). With longer times, tighter caps formed. Because endocytosis occurs at about the same rate as capping, relabeling of surface dextran with fluorescein-labeled rabbit antisheep IgG was carried out to ascertain that these caps were surface structures. They were. We did not observe a measurable increase in cell number during capping. Furthermore, neither the labeling of cells with stearoyldextrans nor the capping of stearoyldextrans caused a measurable change in the rate of cell division during 48 h of observation. Capping also occurred at room temperature but ~10 times more slowly. Leaving the antibodies in solution did not inhibit capping.

While our nonsynchronized, moderately confluent 3T3 cells provided a heterogeneous population, some generalities about the types of caps and their correspondence with cell shape can be made. The most prevalent caps were those which formed over the nucleus on flat and elongated tubular cells. It appeared as though the fluorescent layer moved inward from surrounding regions toward the nucleus. Another distinct form of cap which was found on flat and tubular cells was the cap located as though pushed out on a process. A third form of cap was found on some flat cells where the mottling stage was characterized by the fluorescence being pulled away from, rather than toward, the nucleus. This resulted in the cap forming over the perinuclear region. Rounded cells tended to form caps of the classic crescent shape characteristic of lymphocytes. Often these caps were accompanied by a projection of the cytoplasm, again reminiscent of lymphocyte uropods.

Table IV: Effects of Drugs on Stearoyldextran Diffusion and Capping

drug	effect on diffusion coeff	effect on % recovery	effect on capping
metabolic poisons			
10^{-2} M NaN_3	none		inhibits (combination of all three required for good inhibition)
10^{-2} M NaN_3 plus 10^{-2} M 2-deoxy-D-glucose	none		
10^{-2} M NaN_3 plus 10^{-2} M 2-deoxy-D-glucose plus 10^{-2} M NaF	none	none	
microfilament disrupting drug			
cytochalasin B	none	none	strongly inhibits
microtubule disrupting drug			
colchicine	two- to three-fold decrease	none	no strong effect

To test the requirement of cross-linking for capping, we compared the behavior of cells labeled with AcRSD, AcT_5RSD, and TRSD on the addition of anti-TNP antibodies. Surprisingly, both AcT_5RSD and TRSD capped equally well. AcRSD, however, did not patch or cap; rather, it underwent internalization by the endocytotic process described above. This was also the case for cells labeled with AcT_5FSD and then with rhodamine-labeled anti-TNP Fab fragments. We have studied the effect of drugs on stearoyldextran patching and capping. Drug treatment and dose were the same as those used to study drug effects on stearoyldextran diffusion. Cytochalasin B had no effect on patching but completely inhibited capping. Colchicine had no effect on patching and had no more than a weak effect, if any, on capping. The effects of cytochalasin B and colchicine on mottling were not discernable because of the rounding of cells which results from treatment with these drugs. Metabolic poisons did not affect patching but did inhibit mottling and capping. For nearly complete inhibition of capping, the combination of NaN_3, 2-deoxy-D-glucose, and NaF was required. The effects of drugs on stearoyldextran diffusion and distribution are summarized in Table IV.

(*b*) *Effect on Stearoyldextran Diffusion.* The effect of increasing amounts of anti-TNP IgG on the diffusion and mobile fraction of AcT_5RSD on 3T3 cells at 3×10^3 molecules/μm^2 is given in Table III. In this set of experiments incubations were done at 37 °C in Hanks' BSS, and the measurements were made before significant visible patching or capping occurred and with antibodies remaining in solution.

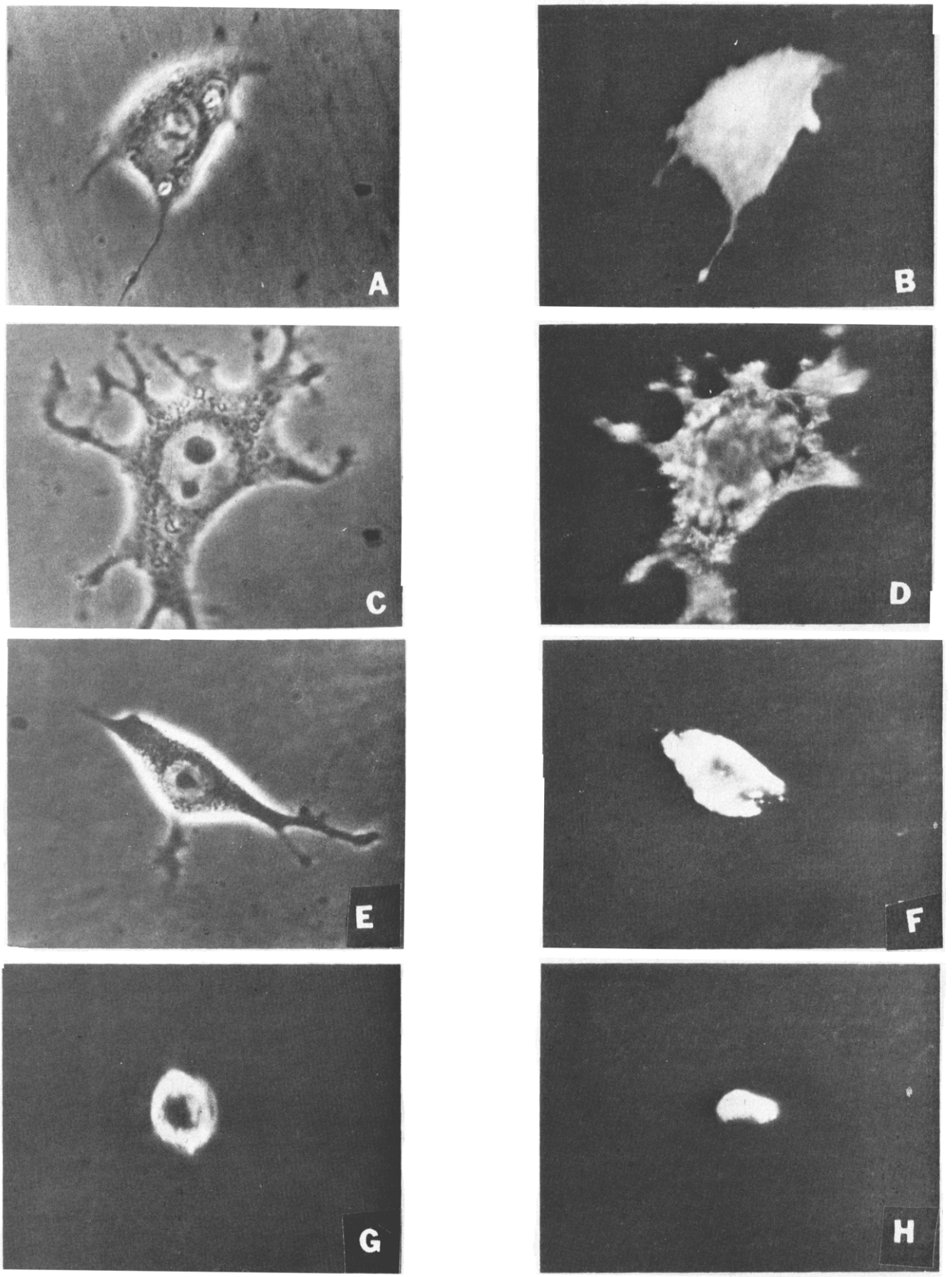

FIGURE 4: Capping of stearoyldextrans on 3T3 cell plasma membranes. (1) Cells were labeled with AcT_5RSD or TRSD at 100 μg/mL for 15 min at 37 °C and then washed. This results in essentially homogeneous fluorescence as shown in (A/B) (phase/fluorescence) for the AcT_5RSD. (2) Cells were then exposed to 25 μg/mL anti-TNP IgG for 30 min at room temperature and then washed. Fluorescence became patchy and mottled with the development of fluorescence-free areas as shown in (C/D) (phase/fluorescence). This patching is similar to that observed by Wolf et al. (1977) for TRSD on BLM. (3) Cells were then incubated at 37 °C for 2.5–3 h which resulted in capping. (E/F) (phase/fluorescence) shows over the nucleus capping of AcT_5RSD, typical of flat and of tubular cells. (G/H) (phase/fluorescence) shows capping of TRSD on a rounded cell. This appearance is identical with that of surface immunoglobulin on B lymphocytes. In (G/H) antibodies were not washed away. These photographs have the same magnification as Figure 3.

The addition of antibodies had two observable effects on diffusion which are similar to those observed on BLM (Wolf et al., 1977): (1) the diffusion coefficient of the mobile fraction decreased by about threefold and (2) the mobile fraction decreased to essentially zero.

The same nearly complete immobilization by antibodies was observed under the conditions used in the patching–capping experiments described above. Before significant visible patching could occur, we found very little recovery, 11 ± 9% for AcT_5RSD and 15 ± 8% for TRSD at room temperature. This immobilization was also observed at 37 °C. Once again we saw no significant dependence on the number of TNP groups. The addition of antibodies to cells labeled with AcRSD or Fab' fragments of anti-TNP IgG cells labeled with AcT_5FSD had no effect on the diffusion coefficient or the fractional recovery. We thus have found that under conditions of cross-linking which result in capping, most of the stearoyldextran diffusion was immobilized prior to capping.

(*5*) *Effect of Stearoyldextran Labeling and Immobilization of Its Diffusion on the Diffusion of Cell Membrane Components.* (*a*) *Effect on Lipid Probe Diffusion.* We have studied the effect of adding stearoyldextran and immobilizing its diffusion prior to capping on the diffusion of the lipid probe dil. Since the excitation and emission spectra of dil precluded separation of its fluorescence from that of either fluorescein or rhodamine, we used nonfluorescent TSD in these experiments. The binding of TSD to cells, immobilization of its diffusion, and subsequent capping on cross-linking were verified by using fluorescein anti-TNP IgG.

For cells labeled with dil alone we found $D = (6 \pm 3) \times 10^{-9}$ cm^2/s with 82 ± 14% recovery. Any incomplete recovery here was due to partial internalization. Complete recovery was observed when measurements were made at a spot on the membrane over cytoplasm free of visible internalized vacuoles. With the addition of TSD at a concentration of 3×10^3 molecules/μm^2, we found for dil that $D = (5 \pm 2) \times 10^{-9}$ cm^2/s with 88 ± 12% recovery. On immobilization of TSD diffusion with anti-TNP antibodies, we found for dil that $D = (7 \pm 3) \times 10^{-9}$ cm^2/s with 86 ± 10% recovery. Thus we have found that neither the addition of TSD nor the immobilization of its diffusion with antibodies had any measurable effect on the diffusion coefficient of dil on 3T3 cells or its fractional recovery. This result is consistent with experiments on BLM (Wolf et al., 1977) and experiments done with natural membrane proteins (Schlessinger et al., 1976b).

(*b*) *Effect of FITC-Labeled Membrane Protein Diffusion.* We have studied the effect of stearoyldextran addition and the immobilization of its diffusion prior to the effect on the diffusion on FITC-labeled membrane proteins. The FITC labeling had no effect on the diffusion of AcT_5RSD or on its immobilization and subsequent capping by antibodies. For cells labeled with FITC alone we found $D = (7 \pm 3) \times 10^{-10}$ cm^2/s with 62 ± 10% recovery. After the addition of AcT_5RSD we found that diffusion of the FITC-labeled proteins was essentially unchanged [$D = (8 \pm 3) \times 10^{-10}$ cm^2/s with 52 ± 11% recovery]. After AcT_5RSD diffusion was immobilized with antibodies but before significant capping occurred, we found that the diffusion of the FITC-labeled proteins was perhaps slightly inhibited [$D = (6 \pm 3) \times 10^{-10}$ cm^2/s with 51 ± 12% recovery]. On those cells which had begun to cap AcT_5RSD, no redistribution of FITC-labeled protein was observed. Thus we have found that neither the addition of AcT_5RSD nor its immobilization with antibodies had any measurable effect on the diffusion and fractional recovery of 3T3 cell membrane proteins labeled nonspecifically with FITC, nor did the FITC-labeled proteins cap with the AcT_5RSD.

(*6*) *Diffusion of Stearoyldextrans on Relabeled Capped Cells.* The nonrecovering fraction found in FPR measurements of the diffusion of some membrane macromolecules raised the question of whether there is a relationship between a protein's fractional recovery and its ability to cap. Recent theories of capping (Ash & Singer, 1976; Bourguignon & Singer, 1977) led us to ask this question differently: Does the capping of stearoyldextrans remove an immobilizing component from the cell surface?

To address this question, we labeled cells with AcT_5FSD and measured its diffusion and fractional recovery. AcT_5FSD was then exposed to anti-TNP antibodies and allowed to cap. The cells were then labeled with AcRSD. Since AcRSD had no TNP groups, there was no danger of it being cross-linked by remaining antibodies. The diffusion and fractional recovery of the AcRSD were measured off the cap. If the capping of the AcT_5FSD removed an immobilizing component from the surrounding membrane, one might expect AcRSD to recover more completely or diffuse more rapidly than AcT_5FSD before capping. On the contrary, however, we observed no differences in the diffusion coefficients and fractional recoveries of the two species. For AcT_5RSD we found $D = (3 \pm 2) \times 10^{-10}$ cm^2/s with 65 ± 17% recovery, and for AcRSD we found $D = (3 \pm 1) \times 10^{-10}$ cm^2/s with 65 ± 9% recovery. AcRSD which labeled within the cap, however, was found like the capped AcT_5FSD to be immobile. This is as expected from similar experiments done on BLM (Wolf et al., 1977) where one stearoyldextran became trapped in the immobile lattice of another. We thus concluded that capping stearoyldextrans did not measurably remove an immobilizing factor from the cell surface.

Discussion

Understanding lateral movements, patching and capping of cell membrane protein receptors are complicated by the diversity of the possible interactions of these molecules with each other and other cell components. Stearoyldextrans provide us with a model system where some of these difficulties may be avoided. Wolf et al. (1977) have shown that on BLM, where interactions with other cell components are impossible, stearoyldextrans exhibit some of the features of the behavior of cell membrane proteins: treatment with cross-linking ligands immobilizes them and can cause them to patch. Thus the patching process need not require cell metabolism, coupling to the cyto- or exoskeletons, or other special biological events. When incorporated into a cell plasma membrane, these molecules take on additional features of cell membrane protein behavior: their diffusion coefficients and immobile fractions slow to values similar to those of membrane proteins, and like some membrane proteins they can be made to cap on cross-linking with antibodies. What can this remarkable correspondence of behavior between such dissimilar molecules tell us about the mechanisms of cell membrane protein movement, in particular, about the mechanisms of patching and capping? We consider these various observations below.

(*1*) *Incorporation of Stearoyldextrans into 3T3 Cell Plasma Membranes.* The mode of binding of stearoyldextrans into the plasma membranes has been investigated by several routes. An implicit hypothesis of our analysis of the stearoyldextran behavior in the cell membrane is that it binds directly to the lipid bilayers by intercalation of the stearoyl groups into the lipid membrane. Binding via the stearoyl tails is clearly established by the observation that without the stearoyl tails these molecules bind neither to cells nor to model membranes (Wolf

et al., 1977). The experiments demonstrate (1) that stearoyl groups do result in binding to bilayers and (2) that without the stearoyl groups the saccharides with chromophores and haptens do not bind to cell membrane proteins or to lipid bilayers. Furthermore, the fact that the same saturation level of $\sim 10^4$ stearoyldextran molecules/μm^2 is observed on BLM, large unilamellar vesicles, and a wide variety of cell types strongly suggests a common mode of interaction which can only be an interaction between the stearoyl moiety and the bilayers (P. Henkart and C. Neels, unpublished experiments).

The only remaining alternative to the expected stearoyl group binding to the lipid bilayer then becomes stearoyl group binding to regions of membrane proteins which lie outside the bilayer. This possibility would require hydrophobic protein regions in the aqueous domain of the cell membrane, which are unlikely. The observation that the same saturation levels are obtained on binding to lipid model membranes and to a variety of cell types shows that binding does not depend on the presence of specific binding to a particular protein or protein class. The evidence thus, again, strongly suggests that stearoyldextrans are bound to the bilayer by intercalation of their stearoyl groups. These results do not show that the stearoyldextrans are necessarily dispersed as monomers or even that all of the stearoyl groups are intercalated into the bilayer. There is, however, some evidence from fluorescence correlation spectroscopy experiments (D. E. Wolf and W. W. Webb, unpublished experiments) that the dextran molecules primarily diffuse independently of each other in BLM.

(2) *Diffusion of Stearoyldextrans on 3T3 Cell Plasma Membranes.* Wolf et al. (1977) found that stearoyldextrans on BLM at concentrations of 10^3–10^4 molecules/μm^2 diffuse at room temperature with $D \simeq (3\text{–}7) \times 10^{-9}$ cm^2/s with complete recovery. On solvent-free, large unilamellar vesicles at similar concentrations, Wolf (1979)) found that stearoyldextrans diffuse with $D \simeq 2 \times 10^{-9}$ cm^2/s again with complete recovery. On 3T3 cell plasma membranes we have found that at room temperature stearoyldextrans at $\sim 3 \times 10^3$ molecules/μm^2 diffuse with $D = (3.0 \pm 1.8) \times 10^{-10}$ cm^2/s with $65 \pm 17\%$ recovery. These observations raise two important questions: Why are stearoyldextrans on cells diffusing slowly as compared to model membranes? Why do they show incomplete recovery on cells but not on model membranes?

A stearoyldextran molecule in a membrane can react with its environment in two ways: its stearoyl groups can interact with other components in the bilayer, and its sugar region can interact with components of the aqueous phase near the surface. Fahey & Webb (1978) have shown that differences in the diffusion of dil on BLM and cells are largely due to solvent effects in the BLM, thus precluding useful comparison of stearoyldextran diffusion on BLM and cells. However, they found that in the solvent free bilayers of large unilamellar vesicles, dil diffusion is comparable to that observed on cells. As noted above, Wolf (1979) obtained similar results on comparing stearoyldextran diffusion on BLM and large unilamellar vesicles. However, even in this solvent-free system the diffusion rates they observed are still 5–6 times faster than we have observed on 3T3 cells. While some of this difference may be due to lipid composition, we think it more likely that the interaction of the large saccharides with cell membrane components slows diffusion in cells. Because our experiments showed no effect of stearoyldextran addition on the diffusion of FITC-labeled membrane proteins, strong interaction between stearoyldextrans and diffusing proteins is excluded. However, both steric inhibition of dextran diffusion by the dense population of membrane proteins and strong interaction with the nondiffusing population cannot be excluded. One could investigate the possibility of saccharide interactions by comparing the diffusion of molecules with varying size saccharide components. Such data do in fact exist for reasonably equivalent molecules. Fluorescein GM1 is a natural ganglioside which has been chemically modified by replacing one of the two lipid tails with a fluorescein group and thus is reasonably equivalent to stearoyldextran with a small saccharide, while dil is reasonably equivalent to stearoyldextran with no saccharide. Reidler (personal communication) has recently compared the diffusion of fluorescein GM1 and dil on BLM and 3T3 cells. He found that fluorescein GM1 diffuses 10 times faster than stearoyldextrans on 3T3 cells, while dil diffuses 20 times faster than stearoyldextran. Thus, interactions between the saccharide region of stearoyldextrans and elements of the cell surface are likely deterrents of stearoyldextran diffusion of 3T3 cells. These interactions need not be specific; stearic hindrances might suffice. GM1 has very little sugar and should be only slightly affected by such interactions. This argument suggests that saccharide interactions may be important determinants of membrane glycoprotein diffusion rather than the fluidity of the lipid portion of the cell membrane. It would be interesting to compare the diffusion of a natural membrane glycoprotein in a cell and reconstituted membrane glycoprotein in a vesicle or liquid crystal.

A second question is raised by these diffusion measurements: Why do stearoyldextrans have a nondiffusing fraction on 3T3 cell plasma membranes? Some possibilities are (1) immobilization by binding to an immobile cell structure, (2) entrapment in deep invaginations or on extended microvilli that increase the distances to be transversed for fluorescence recovery, and (3) endocytosis into immobile structures. In the case of stearoyldextrans on 3T3 cells, binding to some immobile structure seems to be implicated, since the other mechanisms are found to be insufficient. Geometric effects of surface irregularities may be ruled out because (1) 3T3 cells have relatively smooth surfaces in attached tissue culture (Porter et al., 1973) and (2) radical alteration of cell shape and architecture with cytoskeletal-disrupting drugs does not change fractional recovery. Endocytosis is ruled out by the observation that metabolic poisons, inhibitors of endocytosis, do not affect fractional recovery. Furthermore, when membrane-bound stearoyldextran is labeled with fluorescent Fab′ fragments, the same fractional recovery is observed for both the stearoyldextran and Fab′ fragments. If some of the stearoyldextran had been endocytosed, it would have been inaccessible to the Fab′ fragments and different fractional recoveries would have been observed. Again, a comparison with dil and fluorescein GM1 is useful. Neither of these probes has a significant nondiffusing fraction on 3T3 cells (Schlessinger et al., 1976a; Reidler, personal communication). We propose that the large saccharide moieties of the stearoyldextran bind to the nondiffusing structures in the membrane. Schlessinger et al. (1977a) have shown that stearoyldextrans in BLM will bind fibronectin (a major and nondiffusing exoskeletal glycoprotein on some cells). While fibronectin itself is sparse on 3T3 cells, other glycoproteins may be implicated.

(3) *Effect of Cytoskeletal-Disrupting Drugs on Stearoyldextran Diffusion and Fractional Recovery.* It is tempting to attribute incomplete recoveries of stearoyldextrans to the links between the cytoskeleton and cell membrane proteins that have been conjectured to explain the effect of these drugs on capping. However, if this mechanism prevailed, dissolution of these links with drugs should speed up diffusion and increase

fractional recovery. Neither stearoyldextrans nor natural membrane proteins (Schlessinger et al., 1976a,b) are so mobilized. No effect of treatment with cytochalasin or colchicine on fractional recovery has ever been observed, and, where an effect on the diffusion coefficient has been observed, it has been a decrease rather than an increase. In the case of stearoyldextrans we have observed a two- to threefold slowing of diffusion on treatment with colchicine. Such a small effect might be attributed to cell shape changes induced by the colchicine; however, in that case one would expect a similar effect on treatment with cytochalasin B, but this is not observed. It has been proposed that these drugs might alter the fluidity of the bilayer. However, Wolf et al. (1977) have shown that these drugs do not affect the diffusion of stearoyldextrans of BLM. These results, along with the experiments of Schlessinger et al. (1976b) which showed no effect on dil diffusion on cells, rule out this possibility. Ultimately, it must be said that FPR experiments do not provide any evidence in support of direct links with the cytoskeleton as the cause of the observed fractional recoveries. Indirect interaction via an intermediary intrinsic cell membrane glycoprotein is still possible, and, in fact, for a molecule like stearoyldextran, which should not span the membrane, only indirect interactions should be possible.

(*4*) *Effects of Cross-Linking on Stearoyldextran Distribution and Diffusion.* (*a*) *Capping Is Not a Diffusion Process.* In principle, capping can occur as a purely diffusive process (Buas, 1977). The capping of stearoyldextrans on 3T3 cells, however, is not a diffusive process. In order for complete capping to occur by diffusion, all of the molecules must be free to diffuse together to form a cap. In the case of stearoyldextrans on 3T3 cells, this is not the case. Prior to cross-linking a significant fraction of the molecules is not free to diffuse. Upon cross-linking all of the molecules lose their ability to diffuse prior to capping. Furthermore, capping of stearoyldextrans is a metabolically driven process which requires the integrity of the cytoskeleton.

Although capping of natural membrane receptors has also been recognized not to be a diffusive process, capping has nevertheless been taken to be "a manifestation of lateral diffusion in that it is temperature dependent and requires cross-linking ligands" (Schreiner & Unanue, 1976). The patching and capping of membrane components have also been taken as evidence of the fluid-mosaic model, that is, that components are free to diffuse within the plane of the membrane (Nicolson, 1976a,b; Schreiner & Unanue, 1976). Furthermore, differences in the ease of capping have been attributed to differences in the diffusibility of these components (Nicolson, 1976a,b).

(*b*) *Cap Formation Is Preceded by Cessation of Diffusion.* Wolf et al. (1977) have shown that patching and mottling on BLM occur in the absence of macroscopic lateral diffusion. This suggests that diffusion will be required for molecular redistribution only if it is required for cross-linking. If molecules are close enough, they can be cross-linked in the absence of macroscopic lateral diffusion.

Our studies have also shown that in the case of stearoyl dextrans on 3T3 cells, cross-linking by antibodies stops diffusion on a macroscopic scale prior to the large-scale movement involved in capping. We suspect, but have not shown, that this result applies generally to natural membrane receptors that cap. FPR experiments on membrane proteins consistently show nondiffusing fractions. Cross-linking should then bind together freely diffusing and nondiffusing proteins. Thus sufficient cross-linking should eliminate diffusion on a macroscopic scale. This raises the question of whether patching, which results in the clearing of receptors, both diffusing and nondiffusing fractions, from macroscopic areas of the cell surface, is merely the result of a passive diffusion process as suggested by Taylor et al. (1971) or whether other processes such as in the high-concentration patching observed by Wolf et al. (1977) on BLM are involved. Some recent experiments on cells further document this immobilization phenomena. Dragsten et al. (1979) have shown that surface immunoglobulin on B lymphocytes diffuses but has a nondiffusing fraction and that cross-linking with anti-IgG completely inhibits diffusion prior to capping. Eldridge (personal communication) reports similar results with concanavalin A receptors on mouse SV3T3 cells. This result suggests that diffusive immobility, rather than diffusive mobility, might be the necessary prerequisite for capping. This is not to imply that simply stopping receptor diffusion is sufficient to induce capping. We propose that formation of nondiffusing ligand–receptor complexes may (1) be necessary for the nondiffusive capping mechanism to work and (2) trigger this process if it is present.

In this paper and the preceding discussion we have carefully distinguished between diffusible and mobile. The macromolecules of the cell membrane are capable, at least hypothetically, of motions other than random Brownian motion. Capping is a metabolically driven collective motion. The ability of a cross-linked receptor to cap may in no way reflect its ability to diffuse prior to cross-linking. Thus differences in the ease of capping need not be related to differences in the diffusion rate.

(*c*) *Mechanisms of Capping.* Our experiments with 3T3 cells annd those of P. Henkart and C. Neels (unpublished experiments) show that the capping of stearoyldextrans, like the capping of natural membrane receptors, is a metabolically active process that involves the cytoskeleton in some way. Bretscher (1976) has proposed that the capping of surface immunoglobulin on lymphocytes might be explained by a continuous hydrodynamic flow of lipid toward a sink at one pole of the cell. Proteins would maintain their homogeneous distribution by diffusing at a rate fast enough to resist this flow. Patches, however, because of their size would diffuse too slowly to effectively resist this flow and thus would be driven toward the sink where they would collect to form a cap. We do not believe that the capping of stearoyldextrans on 3T3 cells is driven by such a lipid flow mechanism. Such a mechanism would require a diffusion rate, D, sufficiently fast to counter that of the flow velocity, v. Bretscher (1976) considers the one-dimensional case where the concentrations, C_a and C_b, at two points on a cell, a and b, separated by a distance L, are related by the equation $C_a/C_b = \exp(-vL/D)$. The flow velocity would be given by v = capping distance/capping time. Thus for stearoyldextran on 3T3 cells $v \simeq 100\ \mu m/(L \cdot h) = 3 \times 10^{-6}$ cm/s at 37 °C, while $D \simeq 7 \times 10^{-10}$ cm^2/s. Thus for two points, a and b, separated by 100 μm $C_a/C_b = e^{-43} \simeq 0$. Thus stearoyldextran diffusion wouuld not be sufficient to maintain homogeneous distribution against this hypothetical flow. The same conclusion holds at room temperature. Furthermore, we have found that 35% of the stearoyldextrans are neither diffusing nor flowing prior to cross-linking and that, after cross-linking, all of the stearoyldextrans become cross-linked to this fraction and at 37 °C begin to flow to form caps with $v \simeq 10^{-2}$ μm/s.

The observation that stearoyldextrans, like most membrane proteins, have a nondiffusing fraction that prior to capping all of the stearoyldextran becomes cross-linked to this fraction

and is rendered immobile and cytochalasin B inhibition of capping are suggestive of mechanisms which propose direct links with contractile cytoskeletal fibers. As we inferred above, FPR results do not support the existence of such direct linkages with the cytoskeleton, but indirect linkages through intermediaries are a possibility. Recently, P. Henkart (personal communication) has observed that, like natural membrane receptors (Ash & Singer, 1976), stearoyldextran patches align above stress fibers on WI-38 cells. These cells were used because they have unusually prominent stress fibers. Similar observations have been made for a variety of natural membrane receptors (Bourguignon & Singer, 1977; Ash & Singer, 1976). That these alignments can occur for a molecule like stearoyldextran which cannot itself span the bilayer and that such a molecule can cap preclude capping mechanisms triggered by interactions such as those due to conformational changes of the capping molecule which result from ligand binding.

If stearoyldextran is binding to some immobilizing macromolecule in the cell membrane, which in turn is connected to microfilaments which pull the stearoyldextran along with the macromolecule into the cap, then one might expect capping would remove some of this macromolecule from the membrane, and, if the cell were relabeled with stearoyldextran, it would diffuse more freely. As we have seen, such is not the case. Furthermore, P. Henkart and C. Neels (unpublished experiments) have shown that capping of SIg on B cells does not prevent the subsequent capping of stearoyldextrans and that stearoyldextran capping occurs at the same site as SIg capping. Thus capping does not measurably remove from the cell membrane either an immobilizing component or a component responsible for the capping, or else this component is rapidly regenerated.

It may be that the cytoskeleton is responsible for capping but not for observed nondiffusing fractions. If some other structure, possibly the exoskeleton, is responsible for immobile fractions, then it too may or may not be linked to the cytoskeleton.

If the cytoskeleton is attached to the immobilizing structure, then capping could be explained in terms of the theory proposed by dePetris & Raff (1973) where receptors are only intermittently bound to the immobilizing structure. A single molecule can diffuse away during an unbound period, while a patch is unable to diffuse away and is pulled along with the immobilizing structure by the cytoskeleton. It is not necessary to invoke smaller diffusion coefficients to explain the inability of patches to diffuse away. Cross-linking would join together many sites of receptor binding to the immobilizing structures so that a patch would always be bound at many points to the immobilizing structures. The cocapping experiments of P. Henkart and C. Neels (unpublished experiments) could also be explained in terms of this mechanism even if binding to immobilizing structures were permanent, so long as different receptors associated with different immobilizing factors.

If the cytoskeleton is not associated with the immobilizing structure, then capping could result from the cytoskeleton being bound to a membrane macromolecule which becomes attached to the capping protein when the latter becomes patched (Ash & Singer, 1976; Bourguignon & Singer, 1977). This could occur if the macromolecule that is attached to the exoskeleton is simply caught up in the immobile lattice of the patch. The cytoskeleton thus becomes attached to the receptor as a result of cross-linking. In this case, contractions of the cytoskeleton might be resisted by the immobilizing structures, and some of the differences in the ease of capping between receptors might be explained by differences in the degree of association between the receptor and the immobilizing structure or in the ability of the immobile receptor lattice to trap the protein bound to the cytoskeleton. If the immobilizing structure is an exoskeletal protein, then differences in the ease of capping between normal and transformed cells might be explained by differences in the amount or strength of the immobilizing structure after transformation. Fibronectin, for instance, is an exoskeletal glycoprotein found on normal chicken fibroblasts but only in greatly diminished amounts on transformed cells (Yamada et al., 1977). Alternatively, differences in the ease of capping might be due to alternations in the cytoskeletally bound protein which would affect its susceptibility to trapping by the immobilized receptor lattice.

There are, of course, many possible variations on these mechanisms, and mechanisms may vary with receptor and cell type. We feel that a complete understanding of capping will require not only an understanding of how membrane receptors are linked to the cytoskeleton but also what structures are responsible for the observed nondiffusing fractions of membrane components.

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Isolation and Characterization of Normal Rat Kidney Cell Membrane Proteins with Affinity for Transferrin†

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ABSTRACT: Studies were performed to identify membrane receptors for transferrin in cultured normal rat kidney (NRK) cells. Cells were surface iodinated or metabolically labeled with radioactive glycoprotein precursors. Membrane receptors for transferrin were solubilized with the nonionic detergent Triton X-100. The soluble transferrin receptor has been purified ~1500-fold by affinity chromatography using transferrin coupled to Sepharose. Experiments demonstrated that the receptor can be adsorbed to a transferrin–Sepharose gel and be eluted specifically with transferrin. On sodium dodecyl sulfate–polyacrylamide gel electrophoresis, the receptor preparations obtained by one cycle of affinity chromatography display, in addition to components of M_r lower than 20000, a major glycoprotein component of ~170000. Solubilized receptor preparations subjected to two cycles of affinity chromatography revealed a single polypeptide of ~20000 daltons. Further studies indicated that the 20000-dalton polypeptide is a degradation product of the 170000 glycoprotein. Immunological studies showed that antitransferrin antibodies specifically precipitate a transferrin–170000 complex and that a specific antibody against 170000 glycoprotein precipitates the same complex. These results suggest that the 170000 glycoprotein associates with transferrin in specific fashion and that this protein may be a subunit of the transferrin receptor of NRK cells.

Transferrin is a glycoprotein that is responsible for the delivery of iron to various cells (Aisen & Leibman, 1977). This glycoprotein is a factor required for growth and maintenance of some cultured mammalian cells (Bottenstein et al., 1979; Fernandez-Pol, 1978). As with other substances of related function, it appears that the first step of the interaction of transferrin with the cells is the association of the factor with a specific recognition site (receptor) on the surface of the cells (Aisen & Leibman, 1977; Hu & Aisen, 1978). The binding characteristics of this receptor from both red blood cell precursors and placenta have been extensively studied, and it has been shown that the transferrin receptors can be extracted from the cell surface by nonionic or ionic detergents without loss of its ligand binding activity (Aisen & Leibman, 1977; Sly et al., 1978; Hu & Aisen, 1978). More recently, cultured cells, including NRK[1] cells, have been found to possess transferrin receptors (Fernandez-Pol et al., 1979; Hemmaplardh & Morgan, 1974; Larrick & Cresswell, 1979; Hamilton et al., 1979). Little is known on the nature of the transferrin receptors in cultured NRK cells.

In this report we present the binding characteristics of a putative transferrin receptor extracted from NRK cells with detergents. Some properties of the soluble and partially purified receptor and preliminary identification of cell surface proteins interacting with transferrin are also presented.

Materials and Methods

Cell Culture. NRK-B cells (clone 8) were obtained from Dr. R. Ting (Biotech Research, Inc.). Cell stocks were cultured in 75-cm^2 Costar tissue culture flasks in DME containing 10% (v/v) calf serum (Colorado Serum Co., Denver, CO) as previously described (Fernandez-Pol et al., 1977). For experiments, cells were grown in 150-mm Falcon tissue culture

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[1] Abbreviations used: NRK, normal rat kidney; DME, Dulbecco–Vogt modified Eagle's medium; $NaDodSO_4$, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; PBS, phosphate-buffered saline; PEG, poly(ethylene glycol).